APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2008, p. 2924–2927 Vol. 74, No. 9
0099-2240/08/$08.00+0 doi:10.1128/AEM.02871-07

Highly Selective and Rapid Arsenic Removal by Metabolically Engineered *Escherichia coli* Cells Expressing *Fucus vesiculosus* Metallothionein▽

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Received 19 December 2007/Accepted 26 February 2008

An arsenic-chelating metallothionein (fMT) from the arsenic-tolerant marine alga *Fucus vesiculosus* was expressed in *Escherichia coli*, resulting in 30- and 26-fold-higher As(III) and As(V) binding, respectively. Coexpression of the As(III)-specific transporter GlpF with fMT further improved arsenic accumulation and offered high selectivity toward As. Resting *E. coli* cells coexpressing fMT and GlpF completely removed trace amounts (35 ppb) of As(III) within 20 min, providing a promising technology for compliance with the As limit of 10 ppb newly recommended by the U.S. EPA.

Arsenic (As), a metalloid, is a known human carcinogen affecting millions of people worldwide (25, 33). Arsenic exists in two forms: trivalent arsenite [As(III)] and pentavalent arsenate [As(V)]. Exposure to As can result in increased risks of hypertension (5, 6), skin, lung, and bladder cancers (14), and hyperkeratosis (4), due to inhibition of oxidative phosphorylation (11), interference with cell signaling by binding to hormone receptors (12), or generation of reactive oxygen species (19).

Conventional techniques for As treatment are mostly ineffective for the uncharged form, As(III) (9, 37), or at low arsenic concentrations. Recently, bioremediation has been gaining momentum as an environmentally friendly and effective alternative for removal of heavy metals (6, 7, 15, 18, 22, 26). Although metal-chelating peptides such as metallothionein (MT) have been overexpressed in microorganisms for enhanced accumulation of Cd and Cu, almost all such peptides lack specificity for As (1, 2, 20, 29, 31, 34, 35). Specific arsenic accumulation has been reported by utilizing the metalloregulatory protein ArsR (16) or phytochelatins (13, 21, 32). However, enzymatic synthesis and the availability of precursors such as glutathione and γ-glutamylcysteine require actively growing cells and limit the utility of the metal-chelating ArsR and phytochelatins.

Recently, a newly identified MT from an arsenic-tolerant marine alga, *Fucus vesiculosus* (fMT), has been cloned and stably expressed as a fusion protein (24) in *Escherichia coli* and has been shown to bind arsenite with high affinity in vitro (23). However, the utility of *E. coli* cells expressing fMT for As removal has not been reported. Here we report the overexpression of fMT in *E. coli* for enhanced accumulation of both As(V) and the uncharged form, As(III). To remove the bottleneck in As(III) uptake, the As(III) transporter GlpF was coexpressed with fMT, resulting not only in further improvement in As(III) accumulation but also in selectivity for As(III). Even resting cells could remove trace amounts of As(III) within 20 min.

Expression of recombinant fMT and its effect on arsenic accumulation. The fMT gene was constructed by annealing 11

TABLE 1. Oligonucleotides used for constructing the fMT gene

Oligonucleotide	Sequence (5′ to 3′)
1	GAT CGC GGG CAC TGG CTG CAA G
2	ATC TGG GAA GAC TGC AAG TGC GGA GCG GCG TGC AGC TGC GGC
3	GAC TCG TGC ACC TGC GGA ACT GTC AAG AAG GGC ACC ACC TCT CGC GCC
4	GGC GCG GGC TGC CCC TGC GGC CCC AAG TGC AAA TGC ACC
5	GGC CAA GGC AGC TGC AAC TGC GTC AAG GAC GAC TGC TGC
6	GGC TGC GGC AAG TAA CTG CA
7	CTT GCA GTC TTC CCA GAT CT T GCA GCC AGT GCC CGC
8	TCC GCA GGT GCA CGA GTC GCC GCA GCT GCA CGC CGC TCC GCA
9	GCA GGG GCA GCC CGC GCC GGC GCG AGAGGT GGT GCC CTT CTT GAC AGT
10	GCA GTT GCA GCT GCC TTG GCC GGT GCA TTT GCA CTT GGG GCC
11	GTT ACT TGC CGC AGC CGC AGC AGT CGT CCT TGA C

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▽ Published ahead of print on 7 March 2008.

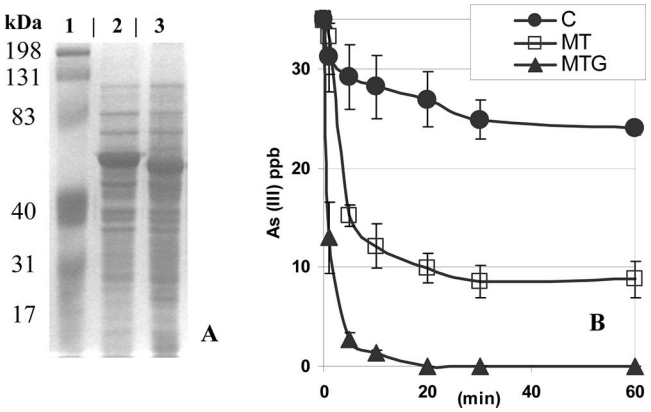

FIG. 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of fMT expression in *E. coli* JM109 harboring either pMALc2x (lane 2) or pMAL-MT (lane 3). Lane 1, molecular weight marker. (B) Removal of 35 ppb of As(III) by resting *E. coli* strain JM109 (5 g/liter) harboring pMAL-c2x (C), pMAL-MT (MT), or pMAL-MTG (MTG). Data are means from three independent experiments. Error bars, standard deviations.

overlapping oligonucleotides (Table 1). The resulting fragment was cloned into BamHI/PstI-digested pUC18 to get pUC18-MT. The fMT gene was then amplified from pUC18-MT, digested with EcoRI/PstI, and ligated into similarly digested pMALc2x (New England Biolabs), allowing the expression of fMT as a fusion with the maltose binding protein for enhanced stability. Figure 1A shows that the fMT fusion protein was detected (~50 kDa) in *E. coli* strain JM109 (27) carrying pMAL-MT by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In contrast, cells carrying pMAL-c2x produced a slightly larger band (~54 kDa) corresponding to the maltose binding protein–β-galactosidase fusion.

To investigate the engineered cells' ability to accumulate As, whole-cell binding experiments were conducted as described previously (16, 36). Cells expressing fMT accumulated levels of As(III) 30-fold higher than those accumulated by the control (Table 2), indicating that the fMT fusions retain their As(III)-binding functionality in vivo. Although only direct in vitro binding of As(III) to fMT has been demonstrated, we were interested in the question of whether the engineered cells could afford similar binding for As(V). Interestingly, the MT-expressing cells also accumulated 26-fold-higher levels of As(V) (Table 2), suggesting that this strain accumulates both forms of arsenic with similar efficiencies. To decipher whether fMT could bind directly to As(V) or whether binding requires prior reduction to As(III), a similar As(V) binding experiment was conducted with the arsenate reductase (ArsC) deletion strain AW10 (10, 17). The level of As(V) accumulation (Table 2) was 10-fold lower than that in JM109, indicating that arsenate reductase is primarily responsible for the reduction of As(V) to As(III), which subsequently binds to fMT. This result suggests that it may be possible to further improve As(V) accumulation by overexpressing arsenate reductase.

Coexpression of MT and GlpF and its effect on As(III) accumulation. To further improve overall As(III) accumulation, the As(III) transporter GlpF (3, 28) was coexpressed with fMT. The synthetic operon was constructed by amplifying the *glpF* gene from pTrc10HisGlpF (Peter Agre, Duke University) using the 5′F primer CGCTGCAGCGGGAGGTCAAT**ATG** AGTCAAACATCAACCTTGA and the 3′R primer TAGTCTGCAGTTAATGGTGATGGTGATGGTGCAGCGAAGC TTTTTG (underlining identifies restriction enzyme sites; boldfacing identifies the start codon); the *glpF* gene was then digested with PstI and ligated into pMAL-MT.

The functionality of the GlpF transporter was demonstrated by observing a threefold enhancement in As(III) accumulation for cells overexpressing GlpF alone over that by the control strain (Table 2); coexpression of fMT and GlpF further increased the arsenic accumulation over that by cells expressing fMT alone. The level of enhancement is consistent with the observed increase in As(III) uptake due to GlpF overexpression (Table 2), reflecting the additive effect on accumulation of coexpression of fMT and GlpF. The final level of 8.1 μmol/g (dry cell weight [DCW]) is three times higher than levels recently reported for other engineered *E. coli* strains (16, 30, 32).

Effects of other metals on arsenic accumulation by growing cells. To investigate the selectivity of fMT toward As(III), binding experiments similar to those described above (16, 36) were performed in the presence of equimolar concentrations of other, competing metals such as Zn, Pb, and Cd. In the presence of Pb (data not shown) or Zn (Table 2), no effect on arsenite accumulation was observed, suggesting that fMT has high selectivity toward As(III). However, As(III) accumulation was reduced by 56% (Table 2) in the presence of Cd, suggest-

TABLE 2. Arsenic accumulation by bacterial cells expressing fMT and the arsenite transporter GlpF

Metal[a]	Final concn (μM)	Arsenic content (μmol/g [DCW]) ± SD[b] in:			
		Control cells[c]	Cells expressing:		
			GlpF	MT	MT + GlpF
As(V)	10	0.27 ± 0.008		6.91 ± 0.393	
As(V)[d]	10	0.07 ± 0.007		0.64 ± 0.054	
As(III)	10	0.20 ± 0.020	0.71 ± 0.029	6.08 ± 0.431	8.61 ± 0.550
As(III) and Zn^{2+}	10 and 10	0.41 ± 0.016		7.59 ± 0.624	
As(III) and Cd^{2+}	10 and 10	0.21 ± 0.032		2.94 ± 0.051	4.51 ± 0.143
As(III)[e]	10	0.18 ± 0.020		4.06 ± 0.235	5.72 ± 0.171

[a] Tested with growing cell cultures unless otherwise indicated.
[b] Based on DCW and three independent experiments.
[c] *E. coli* JM109 cells harboring the control vector pMALc2x.
[d] *E. coli* arsenate reductase deletion strain.
[e] Resting cell culture.

ing that fMT also has high affinity with Cd. Coexpression of GlpF with fMT increased the As(III) level by 30% in the presence of Cd (Table 2), indicating that the As(III)-specific transporter GlpF can be used to improve not only As(III) uptake but also the selectivity of sequestration by taking advantage of the transporter's selectivity toward As(III).

Use of resting cells as As(III) biosorbents. One potential application of the engineered strains is the use of resting cells in the absence of nutrients or antibiotics for arsenic accumulation and removal. Cells were grown as described above, washed, and resuspended in prewarmed TB74S buffer (50 mM Tris, 150 mM NaCl [pH 7.4]). The As(III) accumulation levels were very similar to those achieved with growing cultures (Table 2), suggesting that resting cells have the potential of being used as active biosorbents. Interestingly, cells coexpressing fMT and GlpF showed enhancement of As(III) accumulation similar to that for growing cells, indicating that the GlpF transporter is active even in resting cells.

Since the regulatory limit for arsenic in drinking water has recently been lowered to 10 ppb (i.e., μg/liter) and most chemical methods are ineffective at reducing arsenic levels to these low concentrations, we examined the feasibility of using resting cells for arsenic removal at these low concentrations. At a cell concentration of 5 g (DCW)/liter, cells expressing fMT lowered the As(III) concentration from 35 ppb to 10 ppb within 20 min while cells expressing fMT and GlpF achieved the same reduction within 1 min, with all the added As(III) removed within 20 min (Fig. 1B). In comparison, control cells reduced the As(III) concentration only to 25 ppb, primarily due to nonspecific cell surface adsorption. This substantial improvement in As removal can be attributed to the enhanced uptake provided by the additional As(III) transporter GlpF. More importantly, similar experiments were also carried out with a fivefold excess of Cd, Zn, or Pb, and a minimal effect on As(III) removal was observed (data not shown), demonstrating that these engineered cells have the required selectivity and affinity for As(III) removal and could be a useful biosorbent for water treatment. It is intuitive to suggest that further improvements in As accumulation could be achieved by deleting the As(III) efflux pump (8) in order to eliminate competition with fMT for the free As(III).

This work was supported by grants from the NSF (BES0422791 and BES0329482).

Acknowledgments are due to Peter Agre, Duke University School of Medicine, for providing pTrc10HisGlpF and to B. P. Rosen, Wayne State University, for providing strain AW10.

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